Real Time RT-PCR Analysis of Glucose Utilization Enzymes in Skeletal Muscle of Preterm vs. Full Term Neonates

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Abstract

Preterm birth is a serious and costly health problem, which affects nearly 1 in 8 births in the United States. Intravenous, or parenteral, nutrition is often required in very preterm infants (<33 weeks gestational age), whose digestive tracts are too immature to receive enteral nutrition. Unfortunately, current parenteral feeding methods are also associated with metabolic complications including hyperglycemia, which is a primary factor in postnatal morbidity and mortality. Peripheral insulin resistance is a key factor contributing to hyperglycemia in preterm neonates receiving parenteral nutrition, but the relationship between preterm birth and mechanisms of insulin-stimulated glucose disposal are not well understood. We hypothesized that compared to neonates born full term, preterm neonates possess a diminished capacity for glucose uptake and usage in skeletal muscle. To test this, mRNA transcript amounts were measured in skeletal muscle tissue of preterm and term pigs (delivered on days 106 and 114 of gestation, respectively). Tissue was collected after 6 d of parenteral feeding and transcript amounts for enzymes involved in glucose transport (GLUT1 and GLUT4), glycogen synthesis (glycogen synthase), glycogen degradation (glycogen phosphorylase), and fatty acid oxidation (carnitine palmitoyltransferase I [CPT I]) were determined. Preterm pigs displayed increased ($P < 0.05$) expression of GLUT4, CPT I, and glycogen phosphorylase transcripts relative to term pigs. This suggests that mechanisms for glucose uptake in the preterm neonate are immaturely developed and that muscle cells derive energy more heavily from fatty acids and glycogen stored prenatally as alternatives to circulating glucose. Further investigation of the activity and protein quantities of these enzymes is necessary to validate these conclusions.

Introduction

In the United States, preterm birth (<37 weeks gestation) affects approximately 1 in 8 births (Behrman & Butler, 2007) and in 2002, 65% of all infant deaths occurred in infants born preterm (Mathews et al., 2004). The incidence of preterm birth has increased over 30% since 1981, and in 2005 alone, the estimated cost of medical services related to preterm birth was $16.9 billion (Behrman & Butler, 2007). In spite of this elevated incidence and cost, little is known about the consequences that preterm birth and its associated medical treatments have upon metabolic capacity for nutrient utilization (Green et al., 2005).

Parenteral (intravenous) nutrition is often required in very preterm infants (< 33 weeks gestational age), whose digestive tracts are too immature to receive enteral nutrition. Unfortunately, parenteral nutrition may also predispose neonates to hyperglycemia, which occurs in up to 68% of preterm infants receiving intravenous glucose, but only 5% of term neonates (Mericq, 2006). The incidence and severity of hyperglycemia vary widely based on the infant's degree of developmental immaturity, but it is clear that any level of hyperglycemia is associated with an increased risk of morbidity and mortality among premature infants (Hays et al., 2006).

In recent years, researchers have identified a number of factors that are believed to contribute to the development and maintenance of hyperglycemia in preterm infants receiving parenteral nutrition. These include defective pancreatic insulin secretion, hepatic insensitivity to glucose, decreased abundance of insulin-sensitive tissues, and partial insulin resistance in both hepatic and peripheral tissues (Mitanchez, 2007). It is also now clear that in the preterm neonate blood glucose homeostasis (or the lack thereof) is primarily determined by two important metabolic processes: hepatic gluconeogenesis and insulin-stimulated glucose uptake by peripheral tissues (Rozance, 2011). Much of the clinical research has focused upon the first of

these processes, but persistent hepatic gluconeogenesis has been recognized as a normal characteristic of early postnatal development for the last three decades (Cowett et al., 1983). Accordingly, a wide range of experimental treatments have all proven ineffective in slowing hepatic gluconeogenesis in the preterm neonate (Chacko et al., 2011; Chacko & Sunehag, 2010; Farrag et al., 1997; Sunehag, 2003; Sunehag et al. 1993; Sunehag et al. 1994).

Alternatively, the results of research employing the euglycemic hyperinsulinemic clamp suggest that insulin-stimulated glucose disposal may be a more responsive target for clinical management of glucose homeostasis (Farrag et al., 1997). Indeed, a recent study found that early treatment with intravenous insulin infusion successfully reduced the incidence of hyperglycemia in a relatively large sample of premature infants (Beardsall et al., 2008). Surprisingly, very little research has investigated the relationship between preterm birth and the metabolic pathways underlying insulin-stimulated glucose uptake and utilization.

In healthy individuals, skeletal muscle is the primary site for insulin-stimulated glucose disposal, and within muscle tissue; transport across the cell membrane is the rate-limiting step for glucose metabolism (Shepherd & Kahn, 1999). This process is predominantly dependent upon two transport proteins, which change in concentration at birth. The insulin independent GLUT1 is the predominant transporter during fetal development. At birth, the expression of GLUT1 diminishes quickly and is replaced by the insulin-sensitive transporter, GLUT4. By the end of the first year of life, expression of GLUT1 is undetectable, and GLUT4 is the only glucose transporter observed in human skeletal muscle (Gaster et al., 2000). In the developing fetus, GLUT4 has been observed in muscle tissue as early as 18 weeks gestational age, but it does not exhibit an adult-like pattern of subcellular localization until after birth (Gaster et al., 2000). Additionally, insulin does not become active in the developing fetus until the onset of

corticosteroid action late in the second trimester (Mena et al., 2001). In cases of preterm birth, this developmental timeline suggests that muscle tissue may be inadequately prepared for the transition to primarily insulin-stimulated glucose uptake, but to our knowledge, this relationship has not yet been explored in the research literature.

Similarly, glycogen synthesis does not begin until the second trimester of fetal development, and gradually increases until birth. Accordingly, minimal prenatal glycogen storage is recognized as a primary cause of hypoglycemia in preterm infants that do not receive early parenteral glucose (Mitanchez, 2007). To our knowledge however, no research has examined whether preterm infants also have a diminished capacity to synthesize glycogen relative to infants born full term. As glycogen synthesis is a major destination for glucose disposal following transport into peripheral tissues (Shulman et al., 1990; Woerle et al., 2003), an impairment of this nature could have significant implications for the ability of preterm neonates to maintain glucose homeostasis.

The current study sought to assess differences in the capacity for glucose uptake and utilization in the skeletal muscle of preterm and full term pigs receiving total parenteral nutrition (TPN). mRNA transcript abundance was measured for enzymes involved in glucose transport (GLUT1 and GLUT4), glycogen synthesis (glycogen synthase), glycogen degradation (glycogen phosphorylase), and fatty acid oxidation (carnitine palmitoyltransferase I [CPT I]). It was hypothesized that relative to full term pigs, preterm pigs would exhibit a diminished capacity for glycogen synthesis and insulin-stimulated glucose uptake. These deficiencies may also influence the processes of glycogen degradation and fatty acid oxidation in muscle tissue, as cells may be required to derive a greater proportion of energy from sources other than circulating glucose.

Materials and Methods

Tissue Samples: All procedures were performed using frozen porcine skeletal muscle tissue samples, collected in a previous study (Campbell et al., 2009). In brief, pigs were delivered by cesarean section at 106d (preterm; n=5) or 114d (full term; n=6) of gestation and fed TPN for six days before tissue collection. TPN was formulated to meet all nutrient requirements and infused at rates required to promote moderate weight gain (Table 1).

Table 1. Nutrient Content of TPN Solutions[1]

Ingredients, %	**PUFA emulsion**[2]	**Liposyn II, 20%**	**Aminosyn, 8.5%**	**Dextrose**	**MVI-Pediatric**	**MTE-4**	**Calcium gluconate**
Preterm	0.35	15.2	53	14.4	0.5	0.01	0.075
Full term	0.35	15.2	62.5	20.0	0.5	0.01	0.075

[1] Nutrient contents formulated according to Sangild et al. (2002) and Wykes et al. (1993)

[2] PUFA emulsion contained 12.75% ARASCO, 7.25% DHA-S, 2.5% glycerol, 1.2% egg lecithin, and 0.3% sodium oleate

RNA Isolation: Total RNA was isolated using guanidine isothiocyanate and phenol-based Trizol reagent (Invitrogen). RNA integrity was verified by agarose gel electrophoresis with ethidium bromide staining. Quantity and purity were assessed using spectrophotometric analysis (NanoDrop 1000; Thermo Fisher Scientific) of A_{260} and the $A_{260} : A_{280}$ ratio, respectively.

Primer Development: Intron-spanning primers for GLUT1 (forward: CAGTATGTGGAGCAACTGTGC and reverse: CTTTGGTCTCGGGAACTTTG), GLUT4 (forward: TTTCCAGTATGTTGCGGATG and reverse: CGGGTTTCAGGCACTTTTAG), glycogen synthase (forward: GCTACACGCCAGCTGAATG and reverse:

GAGGGGTCTGCGATGTGTT), glycogen phosphorylase (forward: CCTGTACAAGAACCCGAGAGA and reverse: CCTGGCATACTGGGCAAT), muscle CPT I (forward: TCAAGCCTGTGATGGCTCTA and reverse: CTGCAGTGTGTCTGTGTCCTT), and cyclophilin A (forward: ATGTGCCAGGGTGGTGAC and reverse: AGGACCCGTATGCTTCAGG) were designed from published GenBank pig-specific sequences (Accession numbers GLUT1: EU012358.1, GLUT4: EU590115.1, glycogen synthase: GQ845034.1, glycogen phosphorylase: DQ508264.2, muscle CPT1: AY642381.1, and cyclophilin A: AY008846.1) using Primer3 v 0.4.0 software. Optimal annealing temperature was determined experimentally for each primer set (60.5° C for GLUT4 and glycogen synthase; 59.6° C for GLUT1, glycogen phosphorylase, and CPT I), and similarity of amplification efficiency for cyclophilin A and each target gene was validated.

Real Time RT-PCR: Isolated RNA was reverse transcribed using Omniscript RT (Quiagen) and a mixture of random hexamer and oligoDT primers. Each reverse transcription reaction contained 1.5 µg of total RNA within a final reaction volume of 20 µL. Real Time PCR was conducted using the Applied Biosystems 7500 Real-time PCR system (Applied Biosystems) and SYBR® Green (Quiagen) fluorescence was used for detection of cDNA. 1% uracil-N-glycosylase (UNG) was included in the PCR reaction mixture to eliminate any carryover contamination. After initial incubations at 60° C for 5 min followed by 95° C for 15 min, cycling parameters were 15 s at 94° C, 30 s at gene specific primer annealing temperature, and 35 sec at 72° C for 40 cycles. Fluorescence data was collected at the end of the elongation cycle and a melting curve was programmed and examined for detection of non-specific amplification products. Samples were assayed in triplicate and negative (minus template and minus RT) controls were included in each assay. Final reaction volume was 25 µL containing forward and

reverse primers (Sigma-Aldrich Custom Primers), each at a concentration of 0.3 μmol/L, and 1.0 μL of cDNA template transferred directly from the products of the RT reactions.

Statistical Analysis: Relative transcript amounts were calculated using the $2^{-\Delta\Delta C}{}_{T}$ method (Livak & Schmittgen, 2001) and normalized to cyclophilin A. Values were compared using independent samples t-tests and differences were reported as significant when $P < .05$. SE were calculated using conventional error propagation methods and results are presented as fold differences in transcript expression between preterm and full term pigs.

Results

mRNA transcript abundance for GLUT4, glycogen phosphorylase, and CPT I was increased ($P < .05$) in preterm pigs relative to term pigs. Expression in preterm pigs was 3.58 fold greater for GLUT4 (Figure 1), 4.68-fold greater for glycogen phosphorylase (Figure 2), and 3.5 fold greater for CPT I (Figure 3). Abundance of GLUT1 (Figure 1) and glycogen synthase (Figure 2) transcripts did not differ between treatment groups.

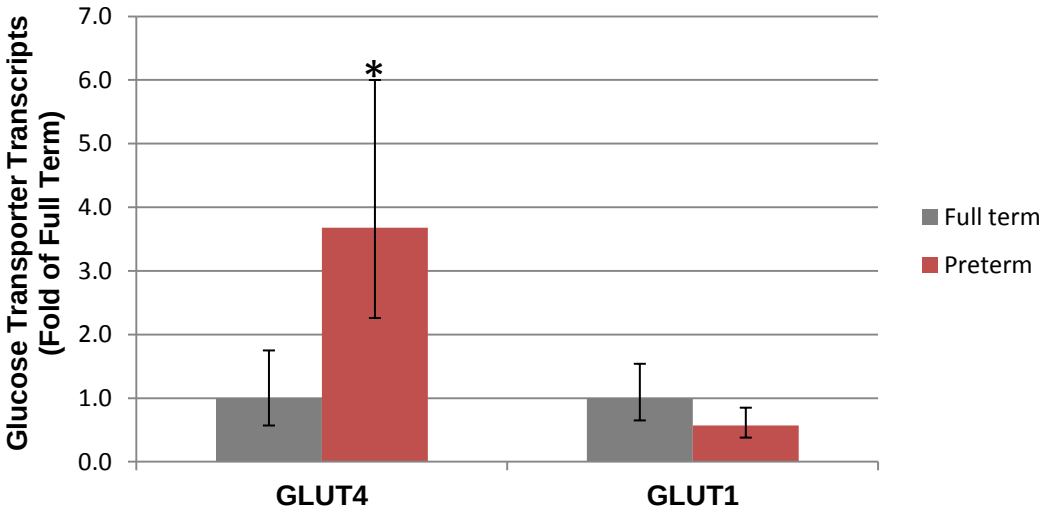

Figure 1 GLUT4 and GLUT1 relative transcript abundance in preterm (n=5) and full term (n=6) pigs. Bars represent mean ± SEM. *Different from the corresponding full term, $P < .05$.

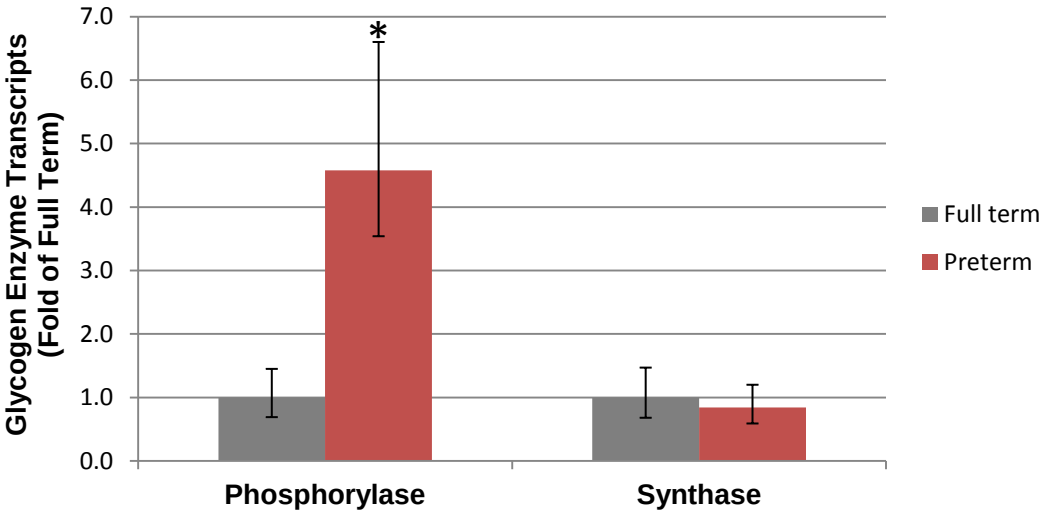

Figure 2 Glycogen phosphorylase and glycogen synthase relative transcript abundance in preterm (n=5) and full term (n=6) pigs. Bars represent mean ± SEM. *Different from the corresponding full term, P < .05.

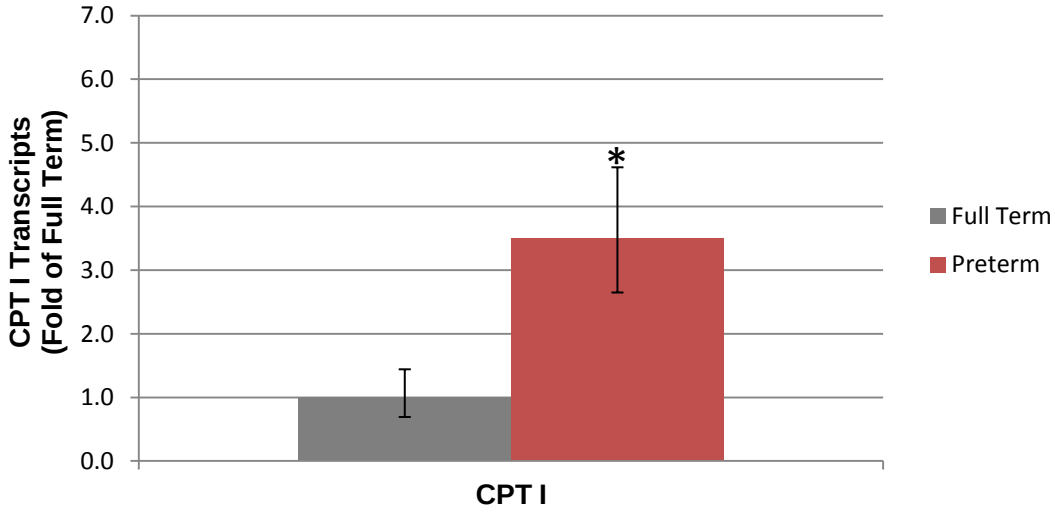

Figure 3 Muscle CPT I relative transcript abundance in preterm (n=5) and full term (n=6) pigs. Bars represent mean SEM. *Different from the corresponding full term, P < .05.

Discussion

The observed differences support our hypothesis that preterm birth is associated with impairment of insulin-stimulated glucose transport in skeletal muscle. Specifically, the elevated GLUT4 transcription in preterm pigs appears to represent a physiological attempt to augment

muscle tissue capacity for glucose uptake from the bloodstream. Two different interpretations seem plausible to explain this association.

As birth is known to mark the approximate beginning of the transition from GLUT1 to GLUT4 mediated glucose uptake, the elevated GLUT4 transcription could be perceived as part of a normal, but maladaptive developmental progression. Because our preterm samples were taken at a time point equivalent to the 112^{th} day of gestation in full term pigs (112 days postconceptional age), the observed increase in GLUT4 transcription could represent an acute change that effectively facilitates the GLUT1-GLUT4 transition in full term neonates. In this case, our results would indicate that this increase in GLUT4 transcription occurs at a specific postconceptional age, insensitive to variation in gestation length. The apparent consistency of the developmental timing achieved by this process is one benefit that could possibly have guided selection for such a system. Additionally, it may be advantageous for the onset of this transition to occur slightly prior to birth, rather than as a reaction to parturition. Unfortunately, this pattern of development would also leave preterm neonates with an immature profile of skeletal muscle GLUT4 expression, and would likely produce a deficiency in insulin-stimulated glucose disposal.

Alternatively, if the timing of the GLUT1-GLUT4 transition is responsive to the event of birth itself, then the observed increase in GLUT4 transcription may instead represent a physiological response to dysregulation of glucose homeostasis in the preterm neonate. In this case, although the mechanisms controlling metabolic development would be sensitive to the attenuated length of gestation, the physical immaturity of the neonate may still result in impaired glucose transport. This could occur due to wide variety of circumstances, but two possible examples would be an absence of sufficient cellular resources to increase GLUT4 expression or

defective function of the signaling pathway that stimulates GLUT4's response to insulin. The latter is already known to be the initial metabolic defect in the development of type 2 diabetes (DeFronzo & Tripathy, 2009), which is a condition that is similarly characterized by peripheral insulin resistance and hyperglycemia. Regardless of the specific nature of the impairment, it is reasonable to expect that the resulting decrease in intracellular glucose concentration would trigger a negative feedback loop, which would ultimately stimulate increased GLUT4 transcription.

Each of the above explanations appears to be plausible within the context of current knowledge about preterm birth and glucose homeostasis in the perinatal period. Additionally, the proposed processes are not mutually exclusive. If low intracellular glucose concentrations do lead to increased GLUT4 transcription, this would be expected to occur regardless of the reason for glucose deficiency. Accordingly, if glucose uptake is impaired in preterm neonates by a developmental delay in the GLUT1-GLUT4 transition, it is likely that both explanations are partially responsible for the observed elevation in GLUT4 transcription. More importantly, *any* of these interpretations implies that the observed difference in the quantity of GLUT4 transcripts indicates a high probability for impairment of insulin-stimulated glucose transport in the skeletal muscle of preterm pigs.

The specific cause of this impairment is less relevant to the central hypothesis of our study, but interestingly, the observed similarity of GLUT1 transcript abundance in preterm and full term pigs does provide some insight into this question. It suggests that the postnatal pattern of GLUT1 transporter expression is unaffected by variation in gestation length, thus providing some evidence that the natural developmental transition from GLUT1 to GLUT4 is equivalently active in preterm and full term pigs of the same chronological age. This is only weak evidence

however, as the size of our sample may not have provided adequate statistical power to detect a real difference in GLUT1 transcription. Although non-significant, our results did indicate that GLUT1 transcripts were nearly twice as abundant in full term versus preterm pigs. This is noteworthy, because this difference is in the direction that would be expected if a decrease in GLUT1 transcription occurs in all pigs, concomitant to the observed increase in GLUT4 transcription, at approximately 112 days postconceptional age. Again, our results suggest that both of these explanations are plausible, underscoring the need for additional research in this area.

The observed elevation of CPT I and glycogen phosphorylase transcription in preterm pigs provides further evidence that glucose uptake is impaired in preterm skeletal muscle. As we hypothesized, these results suggest that these muscle tissues must derive a greater proportion of energy from fatty acids and glycogen due to inadequate concentrations of intracellular glucose. Contrary to our hypothesis, the equivalent quantity of glycogen synthase transcripts observed across treatments suggests that the enzymatic capacity for glycogen synthesis is not impaired in neonates born preterm.

Collectively, the results of the current study provide compelling evidence that insulin-stimulated glucose transport is impaired in the skeletal muscle of preterm neonates. However, the cross-sectional nature of this data provides very little basis for assumptions of causality. A more extensive comparison of these gene transcripts, at additional time points in the perinatal period, could be very helpful in illuminating the true nature of the observed differences. Furthermore, the relationships between quantities of gene transcription, protein expression, and biological activity are not always easily predictable. Before the results of this study can be

linked to any clinically observable phenomena, corroborating evidence is needed from additional research employing techniques such as western blotting and enzyme activity assays.

In spite of these limitations, our results do provide important preliminary findings that should inform future research in this field. To our knowledge, this is the first investigation that has directly examined molecular differences relevant to glucose homeostasis in the skeletal muscle of preterm and full term neonates. The elevated quantities of GLUT4, glycogen phosphorylase, and CPT I transcripts in our sample suggest that this is a promising new direction for investigations of insulin resistance and hyperglycemia in this population. We hope that future research will further examine the relationship between preterm birth and insulin-stimulated muscle glucose disposal, because a better understanding of this topic is clearly needed to inform development of optimal nutritional therapies and minimize the risk of hyperglycemia in preterm infants receiving parenteral nutrition.

References

Beardsall, K., Palmer, C. R., Dunger, D. B., Ogilvy-Stuart, A. L., Ahluwalia, J. S., Midgley, P., Thompson, M., ... Iglesias, I. (2008). Early insulin therapy in very-low-birth-weight infants. *New England Journal of Medicine, 359,* 1873-1884.

Behrman, R. E., Butler, A. S. (2007). *Preterm birth: Causes, consequences, and prevention*. Washington, D.C: National Academies Press.

Campbell, J.A., Martin, J.E., Melendez, K., Stout, M. B., & Lyvers-Peffer, P.A. (2010). Postnatal hepatic fatty acid oxidative capacity of preterm pigs receiving TPN does not differ from

that of term pigs and is not affected by supplemental arachidonic and docosahexaenoic acids. *Journal of Nutrition, 140,*752-759.

Chacko S.K., Ordonez J., Sauer P.J., & Sunehag A.L. (2011). Gluconeogenesis is not regulated by either glucose or insulin in extremely low birth weight infants receiving total parenteral nutrition. *The Journal of Pediatrics, 158,* 891-896.

Chacko S.K., & Sunehag A.L. (2010). Gluconeogenesis continues in premature infants receiving total parenteral nutrition. *Archives of Disease in Childhood: Fetal and Neonatal Edition, 95,* F413-F418

Cowett R.M., Oh W., & Schwartz R. (1983) Persistent glucose production during glucose infusion in the neonate. *Journal of Clinical Investigation, 71*, 467-475.

DeFronzo, R. A., & Tripathy, D. (2009). Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. *Diabetes Care, 32,* 157-163.

Farrag, H. M., Nawrath, L. M., Healey, J. E., Dorcus, E. J., Rapoza, R. E., Oh, W., & Cowett, R.M. (1997). Persistent glucose production and greater peripheral sensitivity to insulin in the neonate vs. the adult. *The American Journal of Physiology – Endocrinology & Metabolism Edition, 272,* E86-93.

Gaster M., Handberg A., Beck-Neilsen H., & Schroder H.D. (2000). Glucose transporter expression in human skeletal muscle fibers. *American Journal of Physiology - Endocrinology and Metabolism, 279,* E529-E538.

Green N.S., Damus K., Simpson J.L., Iams J., Reece E.A., Hobel C.J. ,… March of Dimes Scientific Advisory Committee on Prematurity. (2005). Research agenda for preterm

birth: Recommendations from the march of dimes. *American Journal of Obstetrics and Gynecology, 193,* 626-635.

Hays S.P., Smith E.O., & Sunehag A.L. (2006). Hyperglycemia is a risk factor for early death and morbidity in extremely low birth-weight infants. *Pediatrics, 118,* 1811-1818.

Livak K.J., & Schmittgen T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. *Methods, 25,* 402-408.

Mathews T.J., Menacker F., MacDorman M.F. (2004). Infant mortality statistics from the 2002 period: Linked birth/infant death data set. *National Vital Statistics Reports, 53,* 1-29.

Mena, P., Llanos, A., & Uauy, R. (2001). Insulin homeostasis in the extremely low birth weight infant. *Seminars in Perinatology, 25,*436-446.

Mericq V. (2006). Prematurity and insulin sensitivity. *Hormone Research, 65,* 131-136.

Mitanchez D. (2007). Glucose regulation in preterm newborn infants. *Hormone Research, 68,* 265-271.

Mitanchez-Mokhtari, D., Lahlou, N., Kieffer, F., Magny, J. F., Roger, M., & Voyer, M. (2004). Both relative insulin resistance and defective islet beta-cell processing of proinsulin are responsible for transient hyperglycemia in extremely preterm infants. *Pediatrics, 113,* 537-541.

Rozance, P. J. (2011). Glucose metabolism in the preterm infant. *Journal of Pediatrics, 158,* 6, 874-875.

Sangild P.T., Petersen Y.M., Schmidt M., Elnif J., Petersen T.K., … Burrin D.G. (2002). Preterm birth affects the intestinal response to parenteral and enteral nutrition in newborn pigs. *Journal of Nutrition, 132* 3786–3794.

Shepherd, P. R., & Kahn, B. B. (1999). Glucose transporters and insulin action - implications for insulin resistance and diabetes mellitus. *New England Journal of Medicine, 341,* 248-257.

Shulman G.I., Rothman D.L., Jue T., Stein P., Defronzo R.A., Shulman R.G. (1990). Quantitation of muscle glycogen synthesis in normal subjects and subjects with non insulin-dependent diabetes by ^{13}C nuclear magnetic resonance spectroscopy. *New England Journal of Medicine, 322,* 223-228.

Sunehag A., Gustafsson J., & Ewald U. (1994). Very immature infants (≤ 30 wk) respond to glucose infusion with incomplete suppression of glucose production. *Pediatric Research, 36,* 550-555.

Sunehag A.L. (2003). The role of parenteral lipids in supporting gluconeogenesis in very premature infants. *Pediatric Research, 54,* 480-486.

Sunehag A., Ewald U., Larsson A., & Gustafsson J. (1993). Glucose production rate in extremely immature neonates (< 28 weeks) studied by use of deuterated glucose. *Pediatric Research, 33,* 97-100.

Wykes L.J., Ball R.O., & Pencharz P.B. (1993). Development and validation of a total parenteral nutrition model in the neonatal piglet. *Journal of Nutrition, 123,* 1248–1259.

Woerle H.J., Meyer C., Dostou J.M., Gosmanov N.R., Islam N., Popa E., … Gerich J.E. (2003). Pathways for glucose disposal after meal ingestion in humans. *American Journal of Physiology – Endocrinology and Metabolism, 284,* E716-E725.